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Research Article

PROCESS OPTIMIZATION, PARTIAL PURIFICATION AND CHARACTERIZATION OF PROTEASE ENZYME FROM *BACILLUS ALTITUDINIS* (MCCB-0014)

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ABSTRACT

Protease from a newly isolated strain *Bacillus altitudinis* was partially purified and characterized. The enzyme was purified to homogeneity by ammonium sulphate precipitation. It was found to be able to grow at 40°C and showed proteolytic activity on gelatin agar and some of the physical factors affecting its production were investigated. The enzyme was highly active at an optimum pH of 9.0, incubated for 48 hrs at 40°C and 170 rpm. Optimal conditions for protease production by *Bacillus altitudinis* were found to be at 1% substrate concentration; with 5ml inoculum suspension. Purification of protease resulted in an enzyme with specific activity of 7407.5 (units/mg prot/ml). Purified protease enzyme had a maximum activity at pH 9.0 with Tris-HCL buffer. The microbial growth kinetics was investigated by using Monod's, Andrews and sum kinetic models and found *Bacillus altitudinis* (MCCB-0014**)** under study is a producer of protease enzyme, which can be beneficial for industries.

Keywords: Process Optimization, Purification of Protease Enzyme, Bacillus altitudinis, Growth Kinetics.

INTRODUCTION

Proteases are enzymes which catalyze the hydrolysis of peptide bonds. Proteases are essential constituents of all life forms on earth including prokaryotes, fungi, plants and animals. Proteases are highly exploited enzymes in food, leather, detergent, pharmaceutical, diagnostics, waste management and silver recovery. Proteases (serine protease, cysteine protease, aspartic proteases and metalloprotease) constitute one of the most important groups of industrial enzymes, accounting for about 60% of the total enzyme market. Among the various protease, bacterial proteases are the most significant, compared with animal and fungal proteases and among bacteria, *Bacillus spp.* are specific producers of extracellular proteases[1]. These enzymes have wide industrial application, including pharmaceutical industry, leather industry, and manufacture of protein hydrolisates, food industry and waste processing industry. Thermostable proteases are advantageous in some applications because higher processing temperatures can be employed, resulting in faster reaction rates, increase in the solubility of non gaseous reactants and products and reduced incidence of microbial contaminations by mesophilic organisms. *Bacillus altitudinis* produce a large variety of enzymes, most of which are made in only small amounts and are involved in cellular proteases. Proteolytic enzymes from microorganisms may be located within the cell (intracellular), cell wall associated (periplasmic), or excreted into the media (extracellular). Extracellular enzymes are usually capable of digesting insoluble nutrient materials such as cellulose, protein and starch, and the digested products are transported into the cell where are used as nutrient for growth.

Considering the industrial values of protease, in our present study, we tried to optimize the fermentation conditions for the production of protease from *Bacillus altitudinis* (MCCB-0014) and further tried purify and characterize the enzyme and also tried to determine the growth kinetics.

MATERIALS AND METHODS

Preparation and maintenance of the stock

A culture of *Bacillus altitudinis* (MCCB-0014) previously isolated, taken from MBFT lab of SHIATS. A loopful of bacterial strain were transferred to a tube of sterile nutrient broth and allowed to grow overnight at 37 ºC, at 170 rpm for 24hrs before being used for inoculation. A stock suspension was prepared.

Fermentation procedure

The fermentation medium was prepared by supplementing 100 ml nutrient broth (Purchased from HiMedia Pvt. Ltd., India) in a 250 ml Erylmeyer flask with 10 g w/v gelatin, duly autoclaved at 120°C at 15 lbs pressure for 15 min. The contents of the flask were mixed thoroughly and inoculated with *Bacillus altitudinis* (MCCB-0014) and then incubated for 24 hrs at 37 ºC and 170 rpm in a rotary shaker incubator (Sciegenics Biotech India (P) Ltd).

Extraction of Protease

The whole contents of fermented containing protease were filtered through Whatman No. 1 filter paper to obtain the extracted volume then preserved in the refrigerator at 4º C as a crude protease filtrate [2].

Enzyme assay

The protease enzyme activity was determined by gelatin clearing zone (GCZ) technique. The protease enzyme activity was determined previously [1,7] and standardized later [2-3]. In this assay, soluble gelatine (1% w/v) was emulsified and supplemented with (1.5 % w/v) Bacto-agar and pH was adjusted as required with proper buffer (phosphate buffer at pH 7.0). Cups were made (3 cups optimal) in each plate. Equal amounts (0.1 ml suitable) of extracted enzyme (or enzyme solution) to be assayed were introduced into each cup. The plates were incubated at 35ºC for 24 hrs, at the end of incubation time. The plates were then flooded with previously prepared Mercuric chloride (HgCl) in HCl solution (HgCl- 15g and 20 ml of 6N HCl and maintained the final volume up to 100 ml with distilled water) [3], and the mean diameters of recorded clearing zones were calculated and then expressed in terms of units/ml using a special standard curve constructed for such a purpose [3].

Enzymatic assay of protease

Alkaline protease activity was determined according to the method of Higerd[4]. 1ml of the sample (enzyme) was taken and added to 5ml of 0.6% casein and incubated at 30°C for 10 minutes. The reaction was stopped there by adding 5ml of TCA mixture containing 36 ml of 50% (w/v) TCA solution, 220ml of 1M sodium acetate solution, 330ml of 1M acetic acid solution in a total volume of 1000ml solution. The unreacted casein was precipitated and resulting precipitate was filtered off using Whatman´s No. 1 filter paper. The optical density (O.D.) of sample was taken at 610 nm against appropriate substrate and enzyme blank. A standard curve was generated with pure L-tyrosine as standard. One unit of proteolytic activity is defined as the amount of enzyme, which liberated 1µg of tyrosine in 1 minute under the defined assay conditions. Tyrosine standard solution in the range of 0-1000 mg/ml was prepared in triplicate to obtain a standard curve.

Determination of Protein concentration

Protein content of the sample was the measured by using Bovine serum albumin (BSA) as standard [5]. 0.5ml of enzyme was taken in test tube containing 0.5 ml of 1N NaOH solution. 1ml of reagent was suspended and mixed properly. The mixture was left at room temperature for 10 minutes. 0.5ml of Folin-Ciocalteau reagent was added and mixed. The mixture was left for 30minutes with development of light blue colour. The absorbance of the solution was read at 610 nm.

Enzyme purification

Purification of protease enzyme by ammonium sulfate precipitation was determined using different ammonium sulfate concentration (20, 40, 60 and 80%) in the present study. *Bacillus altitudinis* was grown under optimized conditions. The filtrate broth (crude protease) was collected and centrifuged at 4000 rpm for 15 min at 4ºC in order to obtain a cell free filtrate (CFF). After performing a test for sterility, 200 ml of the cell free filtrate (CFF) containing protease were collected and their proteolytic activities and protein content were determined. 200 ml-1 of the crude protease enzyme were first brought to 20% (w/v) saturation with solid ammonium sulphate (enzyme grade) [6]. The precipitated proteins were regimented by centrifugation for 15 min at 500 min^{-1}. The resulted pellet was dissolved in 5 ml of phosphate buffer at (pH 7.0). The left supernatant was applied again with ammonium sulphate to achieve 20, 40, 60, 80, and 100% (w/v) saturation. Both enzyme activity and protein content were determined for each separate fraction.

Determination of the specific activity of protease enzyme

The specific activity of the protease enzyme protein was expressed in terms of units/mg protein/ml according to the following equation: Specific activity = enzyme activity / protein content (mg/ml).

Optimization of the Fermentation Process

Incubation temperature

The optimum temperature for protease production was determined by taking optical density at 610 nm by inoculating 100 ml growth media containing 1% glucose with the bacterial isolate and incubating it at different temperatures (30, 40, 50, 60 and 70) ˚C.

Inoculum size

The optimum inoculum for protease production was determined by taking optical density at 610 nm by inoculating 100 ml growth media containing 1% glucose with the bacterial isolate and incubating it at different inoculum size (1, 3, 5, 7… 9) ml.

Carbon sources

Each different carbon source (glucose, fructose, xylose, sucrose and maltose) at 1% w/v used for protease production was determined by taking optical density at 610nm by inoculating bacterial isolate and incubating at 35°C.

pH values

The optimum pH for protease production was determined by taking optical density at 610nm by inoculating 100 ml growth media containing 1% glucose with the bacterial isolate and incubating it at different pH (5, 6, 7, 8, 9 and 10).

Incubation period

The optimum incubation period for protease production was determined by taking optical density at 610 nm by inoculating 100 ml growth media containing 1% glucose with the bacterial isolate and incubating it at different incubation period (18, 24, 48, 60 and 72) hrs at 35º C.

Effect of different organic acids

This experiment was carried out in order to investigate the effect of different organic acids (Lactic acid, Acetic acid, Citric acid, HCL and Nitric acid) on purified protease enzyme at same concentration 1% w/v. Protease activity determined by using formula of enzyme activity.

Effect of different amino acids

This experiment was carried out in order to investigate the effect of different amino acids on purified protease enzyme at same concentration 1% w/v. Different amino acids (Arginine, Cysteine, Lysine, Glycine and Tryptophan) were used and the Protease activity determined by using formula of enzyme activity.

Statistical Analysis

Statistical analysis was done by using correlation coefficient and Student's t-test.

RESULTS AND DISCUSSION

Protease production

The extracellular protease enzyme was synthesized by *Bacillus altitudinis* previously isolated. Different culture conditions were used to obtain the maximum levels of protease production by *Bacillus altitudinis* (Fig 1) showed the ability of *Bacillus altitudinis* to utilizing gelatine as a carbon source and energy material to produce protease enzyme. Interestingly, the results indicated that *Bacillus altitudinis* exhibited their maximum ability to biosynthesize protease with 48 hrs incubation period.

The effect of temperature on the protease production was determined and maximum production was obtained at 40˚C (6300 u/ml) and minimum production at 70˚C (3100u/ml) was observed in the present study (Figure 2). Proteinase production was not affected by temperature in the range studied (7-45 ºC).

The effect of size of inoculum on the lipase production by *Bacillus altitudinis* was determined and maximum production was obtained at 5ml (6500 u/ml) and minimum production at 1ml (4600 u/ml) (Figure 3). A lower inoculum may give insufficient biomass causing reduced product formation, whereas a higher inoculum may produce too much biomass leading to the poor product formation.

In the present study the variation in protease production by *Bacillus altitudinis* was determined at different pH (5, 6, 7, 8, 9 and 10). Maximum production was found at pH 9 (6300 U/ml) and minimum by pH 5 (3800 U/ml) was observed in the present study (Fig 4).

Maximum production of protease was obtained at 48 hrs (6400u/ml) and minimum production at 18hrs (4900 u/ml) was observed 36ºC at 170 rpm (Figure 5).

The culture supernatant of *Bacillus altitudinis* containing an initial protease activity (105.5 units/ml) was concentrated by ammonium sulfate precipitation (Fig. 6). The optimum ammonium sulfate fractionation was (80% (w/v) saturation) showed the increase in specific activity. Protease enzyme was purified by ammonium sulfate precipitation. As shown in Ammonium sulfate precipitation resulted in specific activity of 2366.15 (units/mg prot/ml) and purification folds 4 times (Fig. 7). A trial for the purification of protease enzyme resulted in specific activity of 7407.5 (units/mg prot/ml) with purification folds 8 times (Fig. 7).

The specific activity on partial purification of protease enzyme by ammonium sulfate precipitation was determined using different ammonium sulfate concentration (20, 40, 60 and 80%) in the present study. Maximum production was obtained at 80% (7407.5 u/ml) and minimum production at 20% (879.16 u/ml) was observed in the present study (Fig. 8).

The effect of temperature (Fig. 9) on the enzyme activity of purified protease was and found maximum production at 40°C (4800 u/ml) and minimum production at 30°C (3700 u/ml).

The effect of incubation period (Fig. 10) on the enzyme activity was determined and found maximum production was obtained at 48h (5700u/ml) and minimum production at 12h (3800 u/ml).

The effect of pH on the enzyme activity of purified protease was determined using buffer (Sodium phosphate-citrate, Sodium acetate, Tris-HCL and Sodium phosphate buffer) in the present study.

Maximum production was obtained at Tris-HCL (5400u/ml) and minimum production at Sodium Acetate (4000 u/ml) in the present study (Fig. 11).

The effect of organic acids (Fig. 12) on the enzyme activity and maximum production was obtained at nitric acid (5400u/ml) and minimum production at Acetic acid (3800 u/ml).

The effect of amino acids (Fig. 13) on the enzyme activity was determined and aximum production was obtained at tryptophan (5700u/ml) and minimum production at glycine (4200 u/ml).

Fig. 14 showed maximum growth of *Bacillus altitudinis* in growth media containing 10g/l of glucose in 48hrs of incubation period. The decline phase was observed in the microbial growth at both higher and lower incubation period values.

Fig. 15 shows specific growth rate at different concentration of growth media. The best growth was observed at substrate concentration was 10g/l.

The protein estimation and enzymatic assay of protease results were shown in the Fig. 16 and Fig. 17 respectively.

Fig. 1: The clear zone showing the ability of *Bacillus altitudinis* to hydrolyse gelatin and protease enzyme production

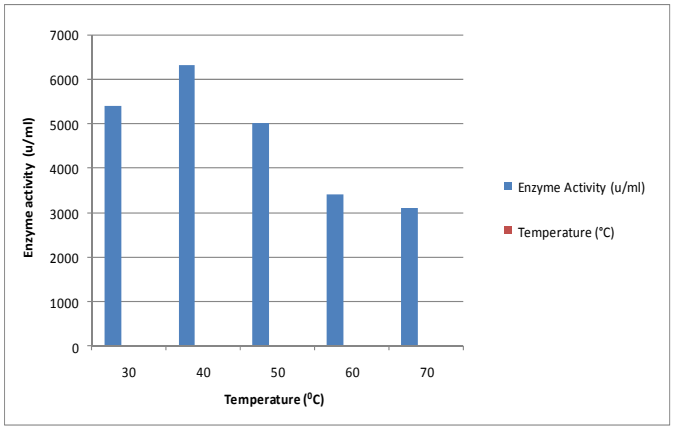

Fig. 2: Protease activities at different temperatures using wheat bran as substrate

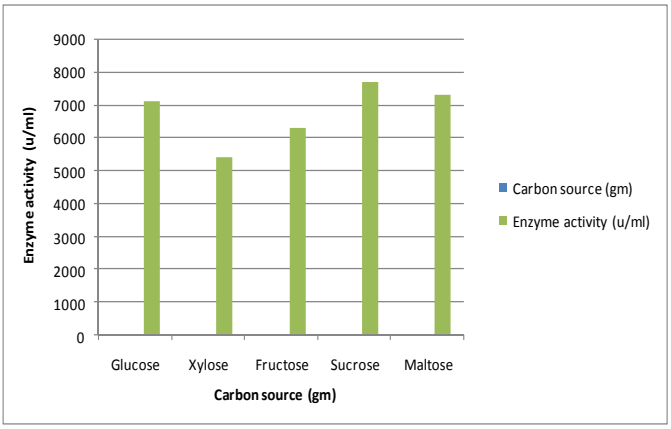

Fig. 3: Protease activities at different carbon sources

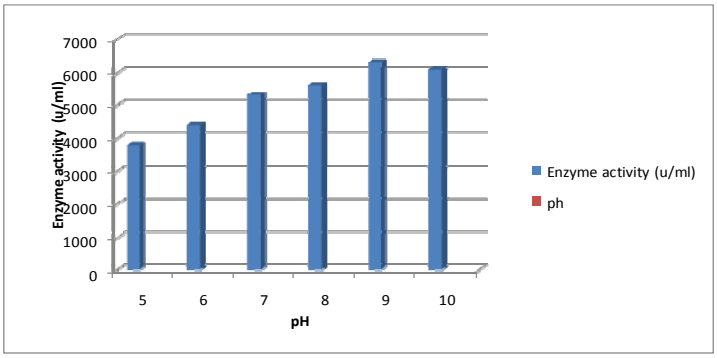

Fig. 4: Protease activities at different pH using wheat bran as substrate

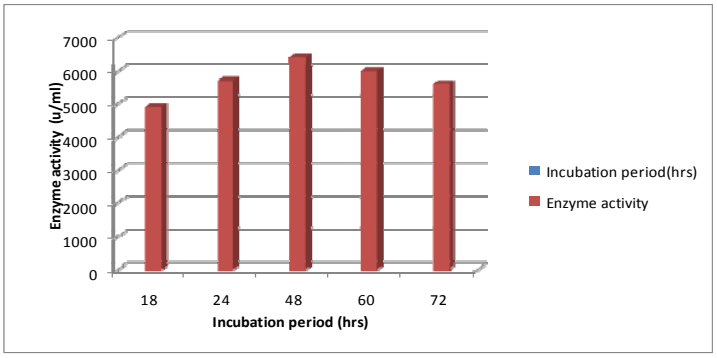

Fig. 5: Protease activities at different incubation period using wheat bran as substrate

Fig. 6: Pellet obtained after ammonium sulphate precipitation

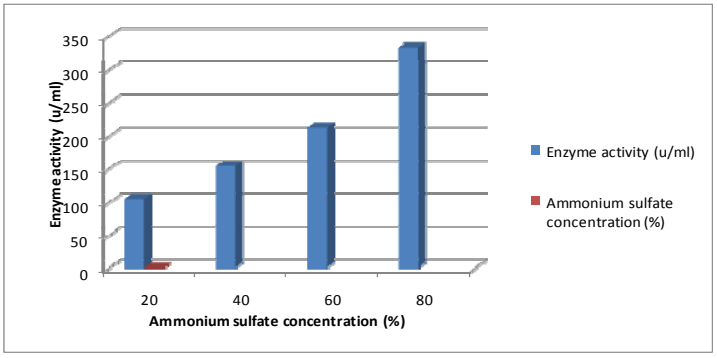

Fig. 7: Protease activities using different concentrations of ammonium sulphate

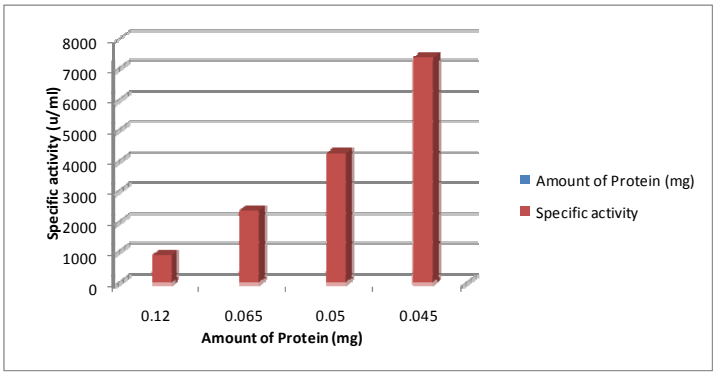

Fig. 8: Enzyme specificities after ammonium sulphate fractionation

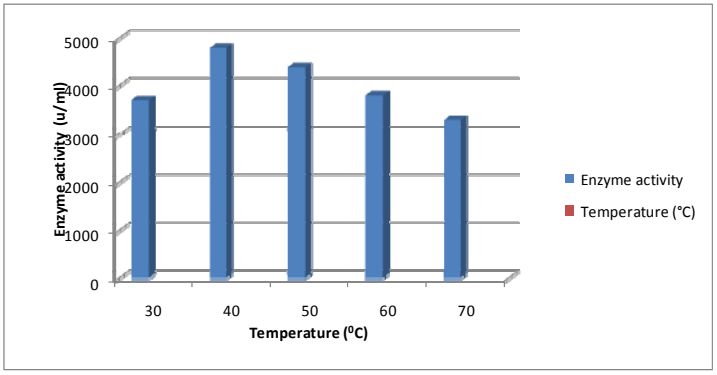

Fig. 9: Protease activities at different incubation temperature

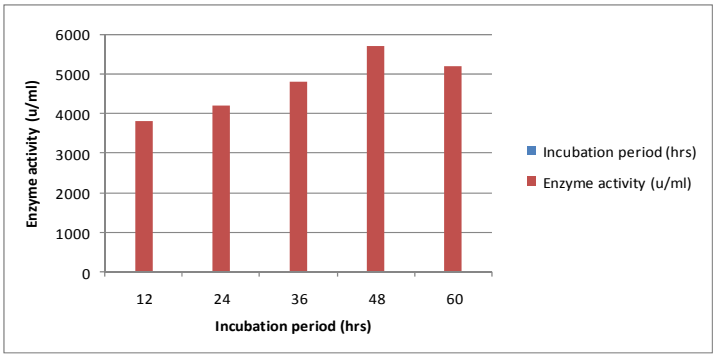

Fig. 10: Protease activities after different incubation periods

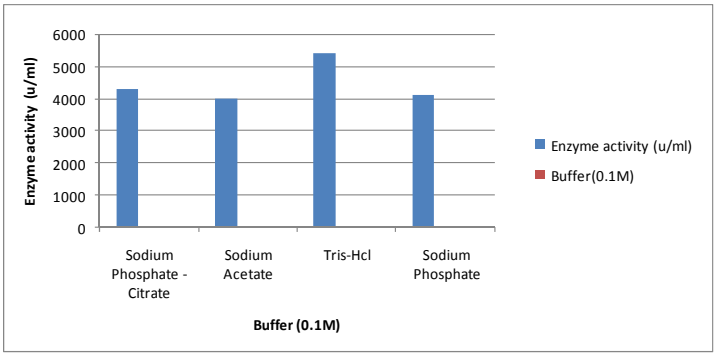

Fig. 11: Protease activities at different buffers

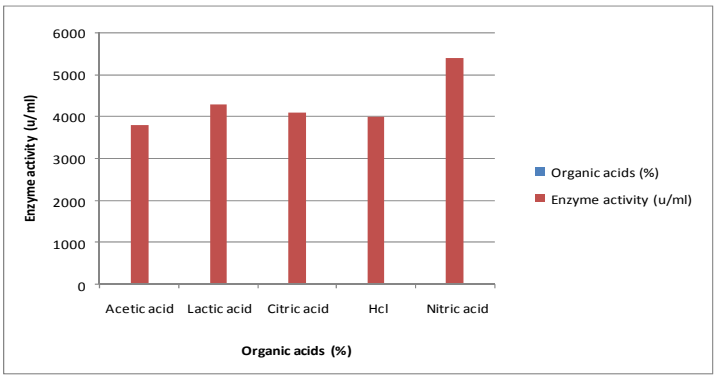

Fig. 12: Protease activities at different organic acids

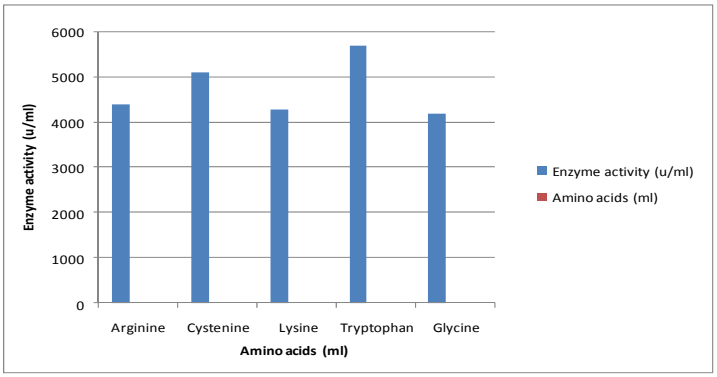

Fig. 13: Protease activities on different amino acids

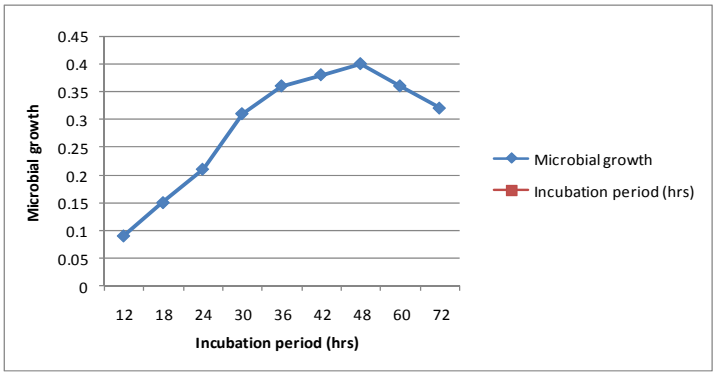

Fig. 14: Microbial growth after different incubation period

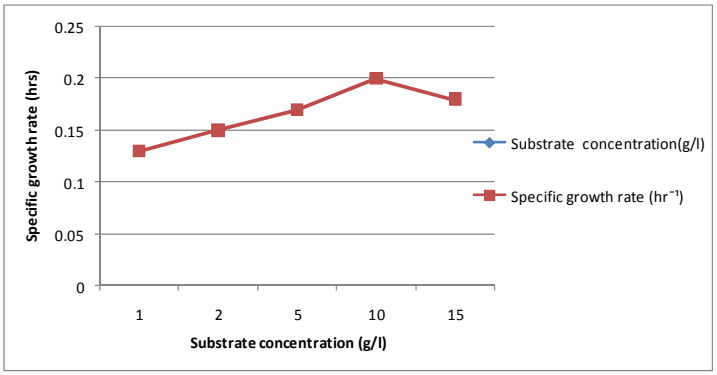

Fig. 15: Specific growth rate at different substrate concentration

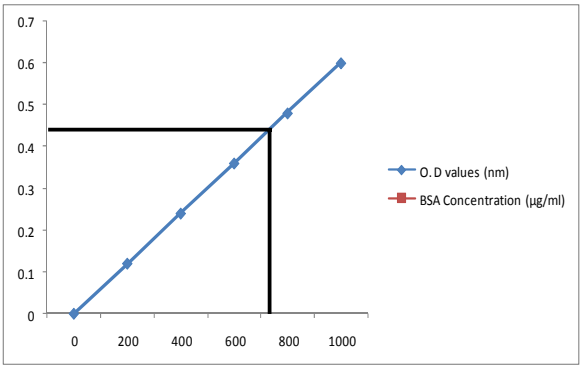

Fig. 16: Determination of protein concentration by Lowry's method

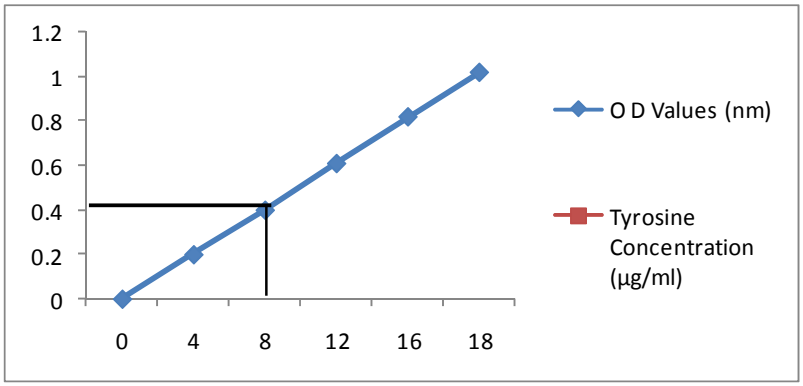

Fig. 17: Determination of enzymatic assay of protease

The results obtained shows that improvement of protease production by *Bacillus altitudinis* strain could be done through selective use of nutrient and growth conditions. Therefore, protease from *Bacillus altitudinis* produced under optimized production conditions, exhibits favorable potential application in food, leather, diagnostics, waste management, silver recovery and pharmaceutical industry. Thus at last it can be said that this enzyme has wide application in various industrial and medical fields and it can be produced in large scale from the microorganism *Bacillus altitudinis* by applying the fermentation technology.

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